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Article

Genomics–Metabolomics Profiling Disclosed Marine *Vibrio spartinae* 3.6 as a Producer of a New Branched Side Chain Prodigiosin

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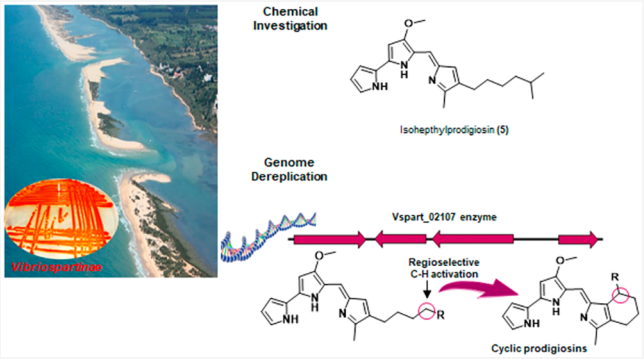
ABSTRACT: A wide range of prescreening tests for antimicrobial activity of 59 bacterial isolates from sediments of Ria Formosa Lagoon (Algarve, Portugal) disclosed *Vibrio spartinae* 3.6 as the most active antibacterial producing strain. This bacterial strain, which has not previously been submitted for chemical profiling, was subjected to *de novo* whole genome sequencing, which aided in the discovery and elucidation of a prodigiosin biosynthetic gene cluster that was predicted by the bioinformatic tool KEGG BlastKoala. Comparative genomics led to the identification of a new membrane di-iron oxygenase-like enzyme, annotated as Vspart_02107, which is likely to be involved in the biosynthesis of cycloprodigiosin and analogues. The combined genomics–metabolomics profiling of the strain led to the isolation and identification of one new branched-chain prodigiosin (**5**) and to the detection of two new cyclic forms. Furthermore, the evaluation of the minimum inhibitory concentrations disclosed the major prodigiosin as very effective against multi-drug-resistant pathogens including *Stenotrophomonas maltophilia*, a clinical isolate of *Listeria monocytogenes*, as well as some human pathogens reported by the World Health Organization as prioritized targets.



The family of tripyrrole red pigments, prodiginines, have attracted considerable research interest over the last few decades due to their wide range of bioactivities, which include antibacterial, antifungal, antiprotozoal, and antimalarial actions.[1] In particular, the immunosuppressant action of natural or synthetic prodiginines[2] has been well investigated, and they have a distinctly different immunomodulatory mechanism than that of cyclosporine. Moreover, they are effective pro-apoptotic agents at nontoxic concentrations.[3−6] Extensive medicinal chemistry optimization of the natural molecules led to the development of a synthetic derivative, obatoclax mesylate (GX15-070), which has been shown to modulate autophagy and has been used in multiple phase I and II combinatorial cancer chemotherapeutics.[7,8] Prodiginines owe their name to their connection with an important miracle of the Christian church, i.e., "The Miracle of Bolsena" (1263): A priest fighting against his decreasing faith received a "prodigious" sign during a Mass when blood started dripping from the holy bread. A few centuries later, the Miracle of Bolsena was explained by the fermentation of the bacterium *Serratia marcescens* on bread, associated with the production of a red pigment.[9,10] Prodigiosin production was first reported for *Serratia marcescens* and then subsequently identified in a variety of terrestrial and marine Gram-positive and Gram-negative microorganisms including *Pseudomonas magnesiorubra*, *Hahella chejuensis*, *Zooshikella rubidus*, *Streptomyces* spp., and *Nocardia* spp.[11−14] Other bacteria such as *Pseudoalteromonas rubra*, *Vibrio gazogenes*, and *Zooshikella rubidus* are able to synthesize cycloprodigiosin in addition to prodigiosin.[13,15,16] Despite the large number of natural producers, there is only minor chemical diversity associated with natural prodiginines. They fall into two broad groups: (1) linear alkyl chain derivatives exemplified by prodigiosin (**1**) and undecylprodigiosin and (2) cyclic derivatives of prodiginines, such as cycloprodigiosin (**2**) and streptorubin B.[2,9,17] The ubiquitous presence of prodiginines in phylogenetically distant bacterial strains seems to indicate a physiological role of these pigments, although the actual role, as for many natural products, is still unclear. The antimicrobial activity of purified prodiginines has been reported in relation to common Gram-positive and Gram-negative bacterial strains such as *Staphylococcus aureus*,

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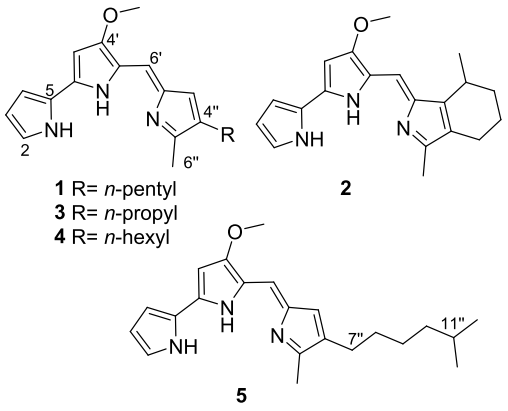
Escherichia coli, and *Bacillus subtilis*.[1,18,19] Analysis of the structure−function relationship of linear and cyclic prodiginines suggests the latter have an enhanced conformational bias toward the interaction with a biological target, which in some cases is associated with increased activity, although no scientific consensus yet exists.[15]

In the context of screening for bioactive metabolites from marine microorganisms, herein, we report on the chemical and biological investigation of a new prodigiosin producer, *Vibrio spartinae*, recently described as a new bacterial species.[20] The bacterium was selected among 59 isolated bacteria from sediments collected in the Ria Formosa lagoon (Algarve, Portugal), for their powerful inhibitory activity exhibited during prescreening for antimicrobial activity against four human pathogens. The study resulted in an almost completely annotated genome of *V. spartinae*, the isolation of both prodigiosin and cycloprodigiosin as major compounds, and the isolation of the first example of a branched-chain prodigiosin, biosynthesized by prodigiosin (*pig*) biosynthetic gene cluster (BGC). Furthermore, through an integrated approach that involved HR-MS^n experiments and comparative genomics, the strain was shown to express an alkylglycerol monooxygenase-like enzyme encoded by Vspart_02107, which is a homologue of PRUB680, recently reported from *P. rubra*. The enzyme likely catalyzes the final cyclization step from prodigiosin to cycloprodigiosin, and it appears to regioselectively catalyze the cyclization of all the linear prodigiosins to their corresponding cyclic derivatives.

A wide-range screening for antimicrobial activity of the isolated molecules revealed that the major prodigiosin is particularly effective against *Listeria monocytogenes*, the causative agent of listeriosis,[21] and *Stenotrophomonas maltophilia*, one of the leading drug-resistant hospital-associated pathogens.[22]



RESULTS AND DISCUSSION

Selection, Identification, and Genome Characterization of the *Vibrio spartinae* 3.6. Recently, in the framework of the EMBRIC Transnational Access program (http://www.embrc.eu/projects/embriceuropean-marine-biological-research-infrastructure-cluster), a total of 24 sediments (Supporting Information, Table S1) were collected from four different points (six replicates each) located in the Ria Formosa lagoon (Algarve, Portugal). The natural park of Ria Formosa is a complex of shallow water and lagoons, known for high fluctuations in tide and high salinity,[23] which makes this environment suitable for the isolation of interesting microorganisms. From these sediments, 59 bacterial strains were isolated and subjected to prescreening for antimicrobial activity on agar plates. The representative pathogenic strains included in the screening were three Gram-negative strains (*Pseudomonas aeruginosa* PAO1, *Escherichia coli* ATCC 25922, and *Acinetobacter baumannii* 13) and one Gram-positive strain (*Staphylococcus aureus* ATCC 6538P). Out of the five active bacteria identified from sediments, the pink bacterial strain labeled 3.6 (isolated from sediment 7B) showed a pronounced lytic halo toward all of the strains with the exception of *P. aeruginosa* (Table S2) and was therefore selected for further investigation. The complete 16S rRNA gene was extracted from the fully assembled genome of *V. spartinae* 3.6 and was compared to the nonredundant (nr) database at NCBI limiting the search to type material. They shared 99.25% similarity to *V. spartinae* SMJ21^T with SMJ21^T=CECT 9026^T=LMG 29723^T.[20] Whole genome sequencing (WGS) of *V. spartinae* 3.6 yielded a genome of 5.0 Mbp distributed between two bacterial chromosomes of 3.8 and 1.2 Mbp, respectively, with a GC content of 45.5%. No further plasmids were detected. Prokka predicted 4320 protein-coding sequences, 90 tRNA and 25 rRNA genes (Table 1).

Table 1. Genome Attributes of *De Novo* WGS of *Vibrio spartinae* 3.6

attribute	value
genome size (bp)	5 010 010
DNA G+C content (bp)	45.5
number of contiguous sequences	2
extrachromosal elements (plasmids)	0
total genes	4520
coding sequences (CDS)	4320
signal peptides	318
tRNA genes	90
rRNA genes (operons)	25 (8)
miscellaneous RNA	84
tmRNA	1
repeat regions	17

To taxonomically delineate *V. spartinae* 3.6, pairwise comparisons were conducted using the Type Strain Genome Server (TYGS), which showed 93% digital DNA−DNA hybridization (dDDH) to *V. spartinae* SMJ21^T, the closest related type strain to *V. spartinae* 3.6. The probability that this value is correct was confirmed by the confidence interval that was between 91% and 94% by linear regression. The threshold for correct taxonomic assignment using dDDH is ≥70% for species classification. Therefore, based on these results it was concluded with a high degree of confidence that isolate 3.6 can be correctly assigned to *V. spartinae*.[24] Here, we see the user strain "Vibrio" is contained within the same species cluster as the type strain *V. spartinae* SMJ21^T=CECT 9026^T=LMG 29723^T (Figure 1).

Primary metabolism of the isolate was reconstructed from genome sequence data (translated amino acids) using KEGG BlastKoala.[25] These data were then used to predict (*in silico*) the biosynthetic pathway of prodigiosin (Figure S1). Thereafter, we confirmed the BGC of *V. spartinae* 3.6 by comparative genomics using the prodigiosin BGC from *S. marcescens* (GenBank accession number AJ833001.1) as the reference. Within the genome of *V. spartinae* 3.6 we discovered the presence of *pigB*−*pigN* as a complete gene cluster, while C-terminal similarity to *pigA* can be found within Vspart_03968

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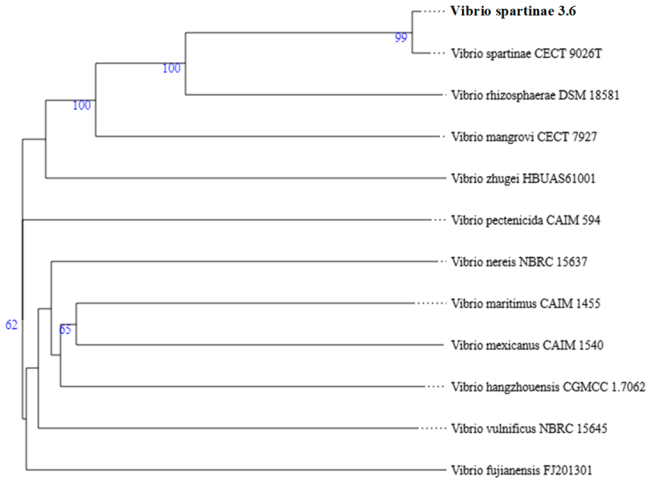

Figure 1. Genome BLAST distance phylogeny (GBDP) by WGS data of *V. spartinae* 3.6. The phylogenetic tree has been inferred from GBDP distances calculated from genome sequences within the Type Strain genome server (tygs.dsmz.de). The numbers below the branches are GBDP pseudobootstrap support values from 100 replications, with an average branch support of 63.6%. The tree was rooted at the midpoint.

located separately on the second chromosome (Figure S2), but this might also be a result of its high sequence similarity to the Acyl-CoA dehydrogenase Adh.

Chemical Identification of Prodigiosins. *V. spartinae* 3.6 was grown in 200 mL of MB mod liquid media at 20 °C for 3 days, and the intracellular and extracellular extracts were mixed, dissolved in mass grade MeOH at a concentration of 1 mg/mL, and analyzed using LC-HRMS in positive mode.

The total ion chromatogram (TIC) shown in Figure 2 highlights the presence of two major peaks, peak B and peak D, respectively, with $[M + H]^+$ of 322.1914 and 324.2071, which are compatible with the pink pigment prodigiosin (**1**), first isolated from *Serratia marcescens*[2] and cycloprodigiosin (**2**), its cyclic homologue, respectively. The remaining peaks, visible in the LC trace—A, C, E, F, and G—showed protonated molecules $[M + H]^+$ at *m/z* 296.1756, 336.2070, 350.2227, 338.2227, and 352.2383, respectively.

The peaks A, F, and G showed molecular weights corresponding to prodigiosin derivatives with C3-, C6-, and C7-alkyl side chains at the 4″ position. Prodigiosin analogues with different alkyl side chains have already been reported in *Zooshikella rubidus*,[13] *Pseudoalteromonas rubra*,[26] and other *Vibrio* spp.,[15] although in some cases the structures were only deduced on the basis of ESIMS data. On the other hand, the molecular weights of peaks C and E indicated one additional unsaturation degree with respect to the peaks F and G.

In order to confirm the results of the LC-HRMS chemical profiling and to assess the antimicrobial activity of the pure prodigiosin components, a large-scale optimized fermentation was established in the same liquid media, and an extract of about 600 mg was prepared and was subjected to repeated solvent partitioning. A preliminary purification by HPLC fractionation gave the major components, prodigiosin (**1**) and cycloprodigiosin (**2**), and three enriched fractions, which were further purified by HPLC to isolate the compounds in peaks A, F, and G. Unfortunately, due to their low abundance and to the presence of some UV-undetectable contaminations, it was impossible to obtain sufficiently purified compounds from peaks C and E for NMR characterization. NMR analysis of the compounds in peaks A, B, D, and F (Table S4) confirmed their identity as 4″-propylprodigiosin (**3**), cycloprodigiosin (**2**), prodigiosin (**1**), and 4″-hexylprodigiosin (**4**).[15]

The absolute configuration of naturally occurring cycloprodigiosin remained unknown for a long time. Only recently, the enantioselective total synthesis of both enantiomers and a combination of X-ray and chiral-phase HPLC analyses allowed

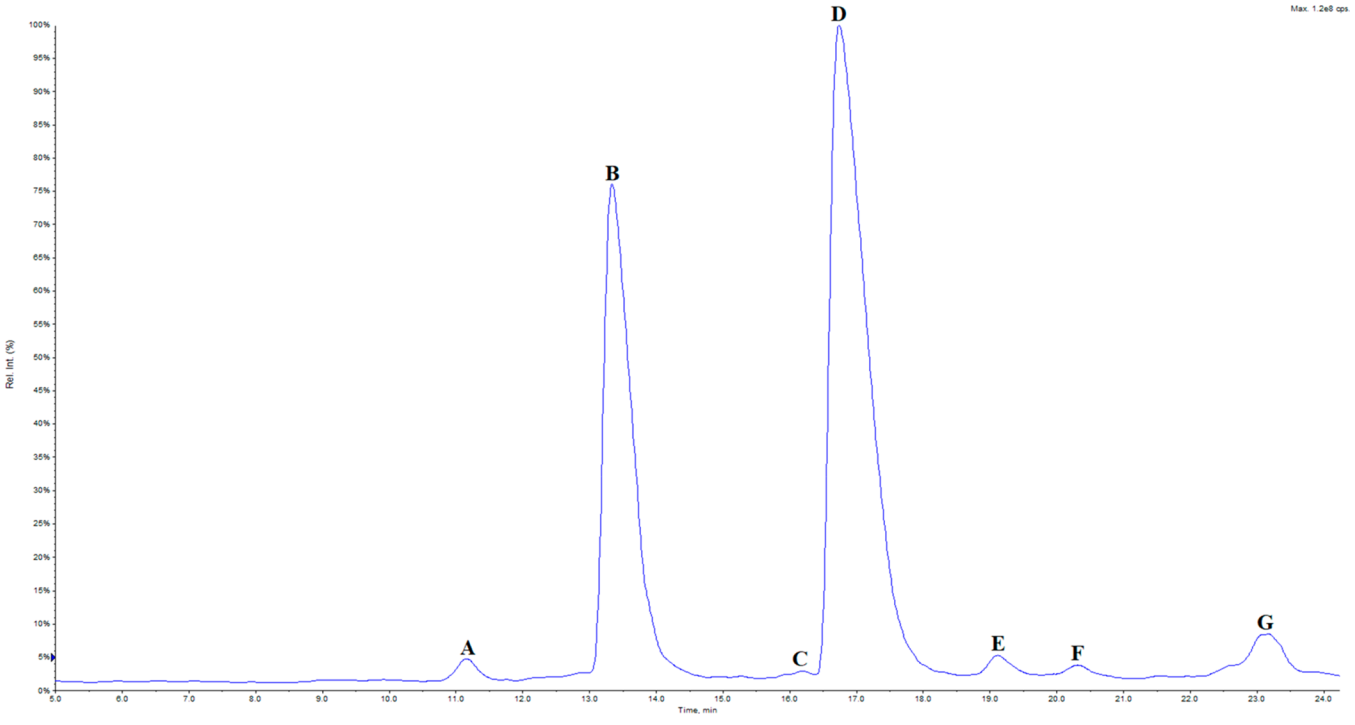

Figure 2. ESI positive mode total ion chromatogram (TIC) of the *Vibrio spartinae* 3.6 MeOH extract.

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for the determination of the natural cycloprodigiosin from *P. rubra* ATCC 29570, as a scalemic mixture occurring in an enantiomeric ratio of 83:17 (*R*)/(*S*).[27]

Accordingly, when we subjected cycloprodigiosin (**2**) from *V. spartinae* 3.6 to chiral-phase HPLC analysis, a comparable ratio was observed (Figure S4).

The molecular formula of compound **5** was deduced as $C_{22}H_{29}N_3O$ based on the protonated molecule $[M + H]^+$ at *m*/*z* 352.2383 in conjunction with ^1H and ^{13}C spectroscopic data. The HRESI MS/MS spectrum showed the fragment ion at *m*/*z* 252.1130, due to the loss of the side chain, which is a fingerprint of the linear prodigiosins, suggesting a core prodiginine structure with a seven-carbon side chain.[28] The ^1H and ^{13}C NMR analysis gave a total match for the signals relative to the prodigiosene nucleus (Table 2), and this was confirmed by 2D NMR analysis. However, in the ^1H NMR spectrum, the usual terminal methyl triplet signal was replaced by a doublet at δ_H 0.88 integrating for six protons. This finding, together with the relative corresponding ^{13}C NMR chemical shift value (δ_C 22.4) as determined by the analysis of the HSQC spectrum, was indicative of an isopropyl terminal subunit. The assignment of the chemical shift values of the seven carbon branched 4″-methylhexyl side chain was straightforward and established by the analysis of 2D COSY, HSQC, and HMBC spectra. For the new compound **5** we propose the name isoheptylprodigiosin.

Table 2. ^1H (400 MHz) and ^{13}C (125 MHz) NMR Assignment of Isoheptylprodigiosin, 5 ($CDCl_3$[a])

isoheptylprodigiosin (**5**)[b]				
position	δ_C, type	δ_H (*J* in Hz)	COSY	HMBC[c]
NH1		12.4, br s	2, 3, 4	
2	127.2, CH	7.25, br s	NH1, 3	3, 4, 5
3	111.9, CH	6.36, br s	NH1, 2, 4	5
4	117.4, CH	6.92, br s	NH1, 3	5
5	121.9, C			
NH1′		12.56[d]	3′	
2′	147.5, C			
3′	92.9, CH	6.08, s	NH1′	2′, 5′
O-Me	58.7	4.01, s		4′
4′	166.3, C			
5′	121.8, C			
6′	116.1, CH	6.95, s	NH1′	4′, 3″
NH1″[b]		12.58[d]	3″	
2″	126.4, C			
3″	128.6, CH	6.67, m	NH1″, 6″, 7″	2″, 5″
4″	128.9, C			
5″	146.9, C			
6″	12.4, CH_3	2.52, s	3″	4″, 5″
7″	25.3, CH_2	2.41, t (7.5 Hz)	3″, 8″	4″, 5″, 8″
8″	30.1, CH_2	1.52[d]	7″, 9″	
9″	26.9, CH_2	1.31, m	8″, 10″	
10″	38.8, CH_2	1.19, m	9″, 11″	
11″	27.8, CH	1.50[d]	12″, 13″	
12″				
13″	22.4, CH_3	0.88, d (6.6 Hz)	11″	11″, 10″

[a]NMR solvent was established for comparative purpose with literature data and to detect exchangeable protons, despite low solubility observed for the compound in this solvent. [b]HPLC conditions furnished pure compound in the protonated form. [c]HMBC correlations are from the proton(s) stated to the indicated carbon. [d]Overlapped with other signals.

There are other examples of branched side chain derivatives in the prodiginine pigment family, i.e., cyclic prodigiosins R1 and R2 and linear 11-methyldodecylprodigininine.[29,30] However, the biosynthesis of the above compounds, isolated from *Streptomyces griseoviridis*, involved the *red* (23 genes) gene cluster, characteristic of Gram-positive *Streptomyces* spp.,[2] which is distinct from the *pig* gene cluster (17 genes) in the Gram-negative *Serratia* spp., responsible for prodigiosin biosynthesis. The isolation of isoheptylprodigiosin (**5**) from the Gram-negative *V. spartinae* 3.6 represents the first report of a branched-chain prodigiosin arising from the *pig* gene cluster. In particular, in the *red* gene cluster, the constitution of an alkyl side chain on the right monopyrrole ring is related to the substrate loading selectivity of RedP, and the replacement by plasmid-based bioengineering of the RedP's function with a *Streptomyces* FAS FabH[31] was reported to produce branched-chain prodiginines related to undecylprodigiosins. On the other hand, in the *pig* gene cluster, the length and the constitution of the side chain on the right methyl-alkyl-pyrrole subunit is influenced by the loading of different 2-alkenoyl CoAs by the thiamine diphosphate-dependent *pigD*.[32] Probably, the *pigD* homologue in *V. spartinae* 3.6 Vspart_01681 is able to load 8-methyl-2-nonenoylCoA, which in turn could be derived from the metabolism of an iso-fatty acid arising from a branched-chain starter unit (Figure 3).

***Vibrio spartinae* 3.6 Is Able to Regiospecifically Catalyze Linear Prodigiosin Cyclization.** Thus far, two unclustered biosynthetic genes have been reported to be responsible for the final transformation of prodiginine linear precursors to their cyclic congeners. In *Streptomyces* spp. the cyclization reaction used to produce cyclic prodiginine derivatives, such as streptorubin B, metacycloprodigiosin, marineosin, prodigiosin R1, and roseophilin, occurs through the action of enzymes belonging to the family of Rieske oxygenases, which are exemplified by REDG in *Streptomyces coelicolor*.[33,34] On the other hand, recent studies on the Gram-negative *P. rubra* DSM 6842 = ATCC 29570 genome[16] disclosed a completely unrelated alkylglycerol monooxygenase-like enzyme, di-iron oxygenase encoded by PRUB680, which was responsible for the regiospecific C10″-H activation and cyclization of prodigiosin to cycloprodigiosin in *P. rubra*.[16] Interestingly, when the whole genome sequence of *V. spartinae* 3.6 was compared to REDG and PRUB680, no match was found with REDG. However, comparison with PRUB680 (GenBank accession no. ERG47138.1) identified a gene, Vspart_02107 (Figure 4), that shared 81% similarity at the amino acid level with PRUB680 based on BLASTP analysis. Moreover, the fatty acid hydroxylase encoded by this gene displayed the conserved histidine motif, which is known to be essential for both iron binding and catalysis,[35,36] and a similar transmembrane topology (Figure S3). Again, the observed incomplete enantioselectivity in the carbocyclization process is another common feature.

A careful analysis of the HR-MSMS fragmentation pattern of the minor prodigiosin-like compounds, which featured one additional unsaturation degree (peaks C and E in Figure 2), revealed the absence of the key fragment *m*/*z* 252.1131 due to the loss of the alkyl side chain at C-4″, a common feature of all linear prodigiosins; additionally, the common fragments C and D+B suggested a common six-membered cycloprodigiosin-like

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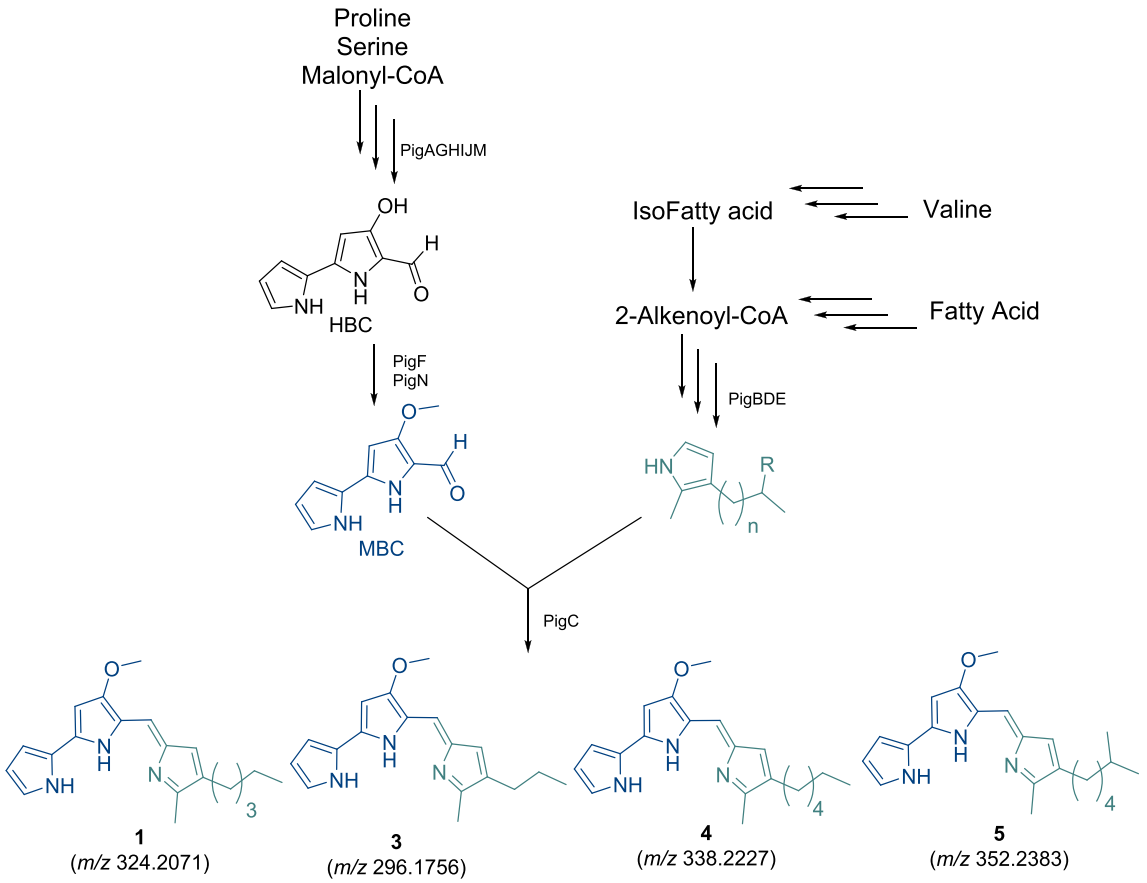

Figure 3. Linear prodigiosins from *Vibrio spartinae* 3.6 (m/z, $[M + H]^+$) and their biosynthetic origins.

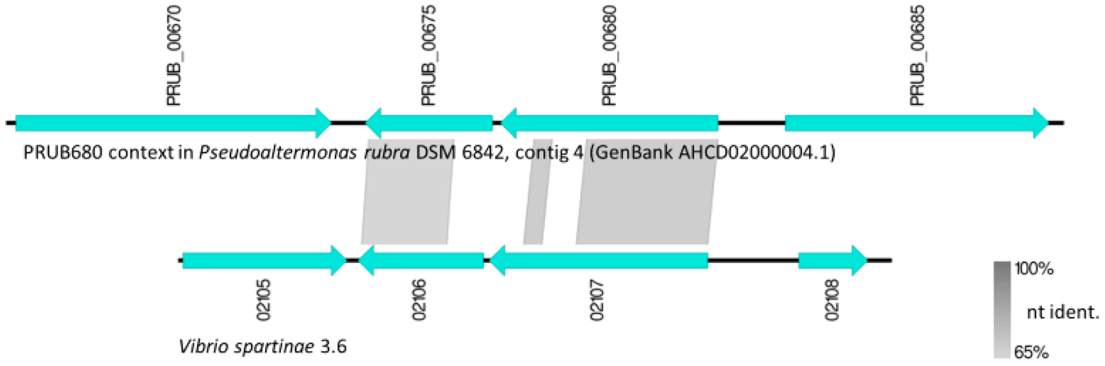

Figure 4. Linear display of PRUB680 and its neighbor PRUB_00675 found in *P. rubra* DSM 6842, in comparison with their homologues in *V. spartinae* 3.6, Vspart_02107, and Vspart_02106.

core, whereas fragments A and D, which differed from each other by +14 amu, were indicative of the presence of homologous side chains at the C-10″ position. Further fragmentation of the daughter fragments A gave the common third-generation fragment m/z 146.0962, due to the loss of the side chain on the cycloprodigiosin core. On the basis of these data, the structures for the compounds correlating to peaks C and E were tentatively assigned as indicated in Table 3, leaving the constitution of the three carbon side chains undetermined, although the iso-propyl chain should be preferred on the basis of biogenetic consideration. This HRMS^n-comparative genomics approach, highlighted the feature of the fatty acid hydroxylase (Vspart_02107) to catalyze the regiospecific oxidative cyclization of all the linear prodigiosins to their corresponding six-membered cyclic derivatives, irrespective of their side chain length. This catalytic capability appears to be a distinctive feature of this enzyme, which is unrelated to previously described proteins involved in the biosynthesis of cyclic prodigiosin-like derivatives.

Antimicrobial Activities of Pure Molecules. As reported in the literature, the data on the antimicrobial activity on prodigiosins, mainly cycloprodigiosin and prodigiosin are dated and very limited.[19] In the present study, we evaluated the antimicrobial potential of the new isoheptylprodigiosin (**5**), together with the major compounds isolated, prodigiosin (**1**) and cycloprodigiosin (**2**), against a wide range of human pathogens. Some of the targeted pathogens used in this assay were from the WHO Priority list of pathogens for which new antibiotics are urgently needed,[37] while others are emergent pathogens. A clinical isolate of *L. monocytogenes* was used

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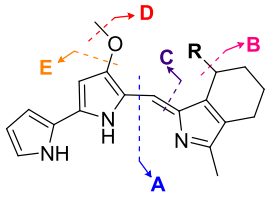
Table 3. HRESIMS Analysis of Peaks B, C, and E



	MS^1 (m/z)	MS^2 fragment ions (m/z)						MS^3 fragment ion (m/z) of fragment A
	R	$[M + H]^+$	fragment A	fragment C	fragment D	fragment E	fragment D+B	
peak B cycloprodigiosin 2	CH_3	322.1914	160.1119	175.0864	307.1676	290.1653	292.1446	146.0962
		$C_{20}H_{24}N_3O$	$C_{11}H_{14}N$	$C_{10}H_{11}N_2O$	$C_{19}H_{21}N_3O$	$C_{19}H_{20}N_3$	$C_{18}H_{18}N_3O$	$C_{10}H_{12}N$
peak C	C_2H_5	336.2070	174.1275	175.0863	321.1832	304.1806	292.1442	146.0961
		$C_{21}H_{26}N_3O$	$C_{12}H_{16}N$	$C_{10}H_{11}N_2O$	$C_{20}H_{23}N_3O$	$C_{20}H_{22}N_3$	$C_{18}H_{18}N_3O$	$C_{10}H_{12}N$
peak E	C_3H_7	350.2227	188.1431	175.0863	335.1986	318.1962	292.1441	146.0961
		$C_{22}H_{28}N_3O$	$C_{13}H_{18}N$	$C_{10}H_{11}N_2O$	$C_{21}H_{25}N_3O$	$C_{21}H_{24}N_3$	$C_{18}H_{18}N_3O$	$C_{10}H_{12}N$

Table 4. MIC and MIC_{50} Values ($\mu g\ mL^{-1}$) for Prodigiosin (1), Cycloprodigiosin (2), and Isoheptylprodigiosin (5) against a Panel of Human Pathogenic Bacteria[a]

	antimicrobial activity ($\mu g/mL$)						
	prodigiosin (1)		cycloprodigiosin (2)		isoheptylprodigiosin (5)		positive control[b]
	MIC	MIC_{50}	MIC	MIC_{50}	MIC	MIC_{50}	MIC
Staphylococcus aureus ATCC 29213	3.3	0.050	4.0	0.060	27	0.50	2.0
Staphylococcus aureus ATCC 23235	1.3	0.060	3.3	0.080	21	0.50	2.0
Staphylococcus aureus 6538P	1.7	0.040	3.3	0.050	21	0.20	1.7
Staphylococcus epidermidis ATCC 35984	2.0	0.33	3.3	0.50	27	2.7	2.0
Listeria monocytogenes MB677	1.7	0.16	4.0	0.40	21	3.3	0.80
Stenotrophomonas maltophilia ATCC 13637	1.7	0.66	3.3	2.0	27	3.3	3.3
Stenotrophomonas maltophilia ATCC 13636	1.7	0.13	3.3	1.0	27	3.3	4.0
Stenotrophomonas maltophilia ATCC 700475	2.7	0.50	5.3	1.7	27	4.0	4.0

[a]Each experiment was repeated at least three times ($n = 3$); the mean value is shown in this table. [b]See Table S3 in the Supporting Information for the antibiotics used as positive controls.

during the antibacterial assay; this bacterium is a foodborne pathogen that is the causative agent of listeriosis, one of the most serious and severe foodborne diseases.[38] This pathogen is developing resistance to many antibiotics commercially in use;[39] particularly, the strain used during this assay was isolated from the cerebrospinal fluid of an infected patient. Three strains of *S. maltophilia* were also used, as it is one of the leading drug-resistant nosocomial-associated pathogens.[22] The majority of the clinical isolate strains have developed resistance to multiple agents used to treat Gram-negative bacterial infections.[40,41] The three pigments showed activity toward both Gram-positive and Gram-negative strains, with prodigiosin (**1**) showing the lowest MIC values (1.3−3.3 $\mu g/mL$) and being approximately 2-fold more active than cycloprodigiosin. The MIC and MIC_{50} values are reported in Table 4.

The MIC values against *Staphylococcus* species conform with previous presented data.[19,42] The antibacterial activity toward *L. monocytogenes* was compared to ampicillin, which is currently used alone or mixed with gentamicin as the drug of choice for listeriosis treatment,[43−45] and the MIC value of **1** was comparable with the positive control. Moreover, the three prodigiosins were tested on three different *S. maltophilia* strains; also in this case, prodigiosin (**1**) displayed the best MIC values (1.7−2.7 $\mu g/mL$). Among the tested compounds, isoheptylprodigiosin (**5**) displayed higher MIC values toward all the tested strains.

It is also worth noting the ability of these pigments to inhibit 50% of bacterial growth at sub-MIC concentrations, in particular of Gram-positive pathogens.

Noteworthy is the antimicrobial activity of **1** against *L. monocytogenes* and *S. maltophilia*, particularly as they are the causative agents of difficult to treat infections that urgently require new antibiotic molecules to counteract them.

Herein, we provide a deep elucidation of the structure−function properties of these novel molecules. In particular, the steric hindrance represented by the presence of a branched chain in the isoheptylprodigiosin (**5**) and of the condensed cycle in the cycloprodigiosin (**2**) negatively affects their inhibitory capacity.

In conclusion, the whole genomic analysis of the marine bacterial strain *V. spartinae* 3.6, isolated from the sediments of the Rio Formosa lagoon in Portugal, and the metabolic pathway prediction revealed the presence of a prodigiosin BGC. Complete dereplication of the metabolic profile by HRESIMS and NMR analysis led to the identification of five prodigiosins, including the first example of a branched-chain prodigiosin derivative arising from a *pig* gene cluster. The production of the branched-chain molecule was assigned to the peculiar substrate flexibility of the *pigD* homologue in the prodigiosin biosynthetic gene cluster of *V. spartinae* 3.6.

Analogously, the presence of two further homologues of cycloprodigiosin together with a high % of cycloprodigiosin

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(more than 50% w/w compared with prodigiosin) was associated with the presence in the *Vibrio* BGCs of a gene encoding for a new member of the alkylglycerol monooxygenase-like enzyme, related to PRUB680 in *P. rubra*. Further investigation of the catalytic properties of this enzyme could expand the biochemical toolbox for the chemoenzymatic transformation of linear precursors of natural and unnatural molecules into their cyclic counterparts via sp^3 C−H activation, a remarkable process that is often not accessible via conventional synthetic methods.

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were recorded with a a Varian Cary 1E UV/vis double ray spectrophotometer (Agilent), in MeOH + 0.1% (v/v) trifluoroacetic acid (TFA) at room temperature. 1D and 2D NMR experiments were recorded on Varian Inova 700 (Agilent) and Bruker Avance NEO 400 spectrometers with an RT-DR-BF/1H-5 mm-OZ SmartProbe. Chemical shifts were reported in δ (ppm) and were referenced to the residual $CHCl_3$ as internal standards (δ_H 7.26 and δ_C 77.0).

The LC-HRMS analysis were carried out on an LTQ XL liquid chromatography high-resolution mass spectrometry system (LC-HRMS) (ThermoScientific) equipped with an Accelera 600 pump HPLC. Purification was performed using a Jasco PU-2089 Plus quaternary gradient pump connected to a UV-2075 Plus UV/vis detector equipped with a Waters Rheodine injector for the first purification step and an Aquity UPLC H-CLASS connected to a PDA detector (Waters) for the final purification of the minor components. The 96-well plates were read on a Biotek ELX800, monitoring the absorbance at 600 nm at room temperature.

Media and Buffers. All reagents and consumables used in preparation of media were purchased from Conda, Sigma-Aldrich, Merck, or PanReac unless otherwise stated. Media were prepared in grams per liter of ddH_2O according to the manufacturer's instructions and autoclaved at 121 °C at 15 psi. For the solid media, bacteriological agar was added at 1.7% (w/v).

Cation-Adjusted Mueller-Hinton Broth (CAMHB).[46] *Marine Broth (MB):* 19.4 g NaCl, 8.8 g/L $MgCl_2$, 5 g/L peptone, 3.24 g/L Na_2SO_4, 1.8 g/L $CaCl_2$, 1 g/L yeast extract, 0.55 g/L KCl, 0.16 g/L $NaHCO_3$, 0.10 g/L Fe(III) citrate, 0.08 g/L KBr, 0.034 g/L $SrCl_2$, 0.022 g/L H_3BO_3, 0.008 g/L Na_2HPO_4, 0.004 g/L sodium silicate, 0.0024 g/L NaF, 0.0016 g/L NH_4NO_3

Tryptone Soy Broth (TSB): 3 g/L papaic digest of soya, 2.5 g/L D-(+)-glucose, 17 g/L pancreatic digest of casein, 2.5 g/L K_2HPO_4, 5g/L NaCl.

Marine Broth modified (MB mod): MB + 10 g/L peptone + 0.3 g/L K_2HPO_4.

Luria−Bertani (LB): 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl.

Nutrient broth (NB): 15 g/L peptone, 6 g/L NaCl, 3 g/L yeast extract.

Bacterial Strains Isolation. The bacterial strains were isolated from four sediment sample sites, and six replicates were collected from each site of the Ria Formosa lagoon (Faro, Portugal) and stored at −80 °C until analysis; location coordinates and environmental features are described in Table S1. The rationale for sediment collection was to obtain samples from different environmental conditions and at different depths. For the isolation of bacteria, one gram of each sediment was gently mixed with 3 mL of sterilized water, and the supernatant was serially diluted (10^{-1} to 10^{-3}) in sterilized water. A 100 μL amount of each dilution was plated onto MB and TSB agar plates. After 20 days of incubation at 20 °C, 59 morphologically different CFUs were selected and inoculated into MB and TSB liquid media.

Screening for Antimicrobial Activity. A single CFU of each of the 59 isolates was inoculated into two 96-well plates, the first filled with 200 μL of MB and the second with TSB, and incubated for 2 days at 20 °C under constant agitation at 120 rpm. Then, the plates were replicated using a pin replicator into five deep-well plates and filled with 1.6 mL per well of five different media: MB, MB mod, TSB, NB, and LB. Finally, the deep wells were incubated at 20 °C for 5 days, under gentle agitation at 120 rpm. After 5 days, each deep well was replicated onto LB agar plates inoculated with a target pathogenic strain at a concentration of 0.04 OD_{600}/mL. *Pseudomonas aeruginosa* O1,[47] *Escherichia coli* ATCC 25922,[48] *Staphylococcus aureus* ATCC 6538P,[49] and *Acinetobacter baumannii* 13[50] were used for these growth inhibition assays.

For the assessment of antimicrobial activity, the plates were inoculated at 20 °C for 24 h to allow the growth of the 59 bacteria. Subsequently, the plates were moved to 37 °C for 24 h to allow the growth of the pathogens, and finally, the active Ria Formosa strains were revealed by the formation of an inhibition halo.

***De Novo* Whole Genome Sequence of *Vibrio spartinae* 3.6.** DNA was isolated using Qiagen Genomic-tip 100/G according to the instructions of the manufacturer. A SMRTbell template library was prepared according to the instructions from PacificBiosciences, following the Procedure & Checklist − Greater Than 10 kb Template Preparation. Briefly, for preparation of the 15 kb libraries, 8 μg of genomic DNA from strain 3.6 was applied unsheared. DNA was end-repaired and ligated overnight to hairpin adapters applying components from the DNA/polymerase binding kit P6 from Pacific Biosciences. Reactions were carried out according to the manufacturer's instructions. BluePippin size-selection to greater than 4 kb was performed according to the manufacturer's instructions. Conditions for annealing of the sequencing primers and binding of polymerase to a purified SMRTbel template were assessed with the calculator in RS Remote, PacificBiosciences. One SMRT cell was sequenced per strain on the PacBio *RSII* taking one 240 min movie. Libraries for sequencing on the Illumina platform were prepared applying the Nextera XT DNA library preparation kit with modifications.[51] Samples were sequenced on NextSeq 500. Genome assembly was performed by applying the RS_HGAP_Assembly.3 protocol included in SMRT Portal version 2.3.0 applying a target genome size of 10 Mbp. Error correction was performed by mapping the Illumina short reads onto finished genomes using the Burrows−Wheeler Aligner bwa 0.6.2 in paired-end (sample) mode using default settings,[52] with subsequent variant and consensus calling using VarScan 2.3.6.[53] Automated genome annotation was carried out using Prokka.[54] The genome has been deposited at NCBI GenBank under accession nos. CP046269 and CP046268.

Species Delineation of *Vibrio spartinae* 3.6 by *In Silico* Type Strain Genome Server. The genome sequence data were uploaded to the TYGS, a free bioinformatics platform available at https://tygs.dsmz.de, for whole genome-based taxonomic analysis.[55] The results were provided by the TYGS on Oct 2, 2019.

Primary Metabolism Analysis by KEGG BlastKoala. The amino acid sequences derived from the nucleotide sequences of the *V. spartinae* 3.6 genome were analyzed by KEGG BlastKoala by selecting "Taxonomy group: Prokaryotes, Bacteria" and the KEGG database searched: "species_prokaryotes.pep" and other default parameters.

KOALA (KEGG orthology and links annotation) is KEGG's internal annotation tool for K number assignment of KEGG GENES using SSEARCH computation. BlastKOALA assigns K numbers to the user's sequence data by BLAST searches against a nonredundant set of KEGG GENES.[25]

Strain Cultivation and Metabolite Extraction. A single CFU of *V. spartinae* 3.6 was used to inoculate 3 mL of liquid MB mod in a sterile bacteriological tube. After 48 h of incubation at 20 °C at 180 rpm, the preinoculum was used to inoculate 200 mL of the same media, at an initial optical density of 0.01 at 600 nm. The flask was incubated for 3 days at 20 °C under constant agitation of 180 rpm. Metabolites were extracted with acetone and EtOAc from the biomass and exhausted broth, respectively; then they were mixed together and evaporated and the obtained extract was dissolved at 1 mg/mL of LC-MS grade MeOH. Finally, 4 μL of extract was injected to carry out the chemical profiling. LC-HRMS dereplication utilized the LC-HRMS instrumentation equipped with an Acquity UPLC BEH C18 1.7 μm column (Waters). The mobile phase A was composed of 100% LC-

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MS mass grade H_2O, and the mobile phase B was composed of 100% MeCN; both phases were added with 0.1% of LC-MS grade formic acid.

Isolation and Purification of Compounds. Large-scale fermentation was obtained by inoculating 1.8 L of MB mod. Pigments were extracted with the same methodology described above, and in addition to that, the extract was subjected to a first hexane/MeOH liquid−liquid partitioning (3 × 100 mL), followed by $CHCl_3/H_2O$ extraction (3 × 100 mL). Finally, the organic layer was dried over anhydrous sodium sulfate, concentrated under reduced pressure, and lyophilized to give about 300 g of dark extract. The extract was subjected to a first HPLC fractionation on a Phenomenex Luna column (5 μm, 10 mm i.d. × 250 mm) using a gradient program (flow rate 0.3 mL/min; 50 μL injection volume). The mobile phase consisted of 0.1% TFA in H_2O (buffer A) and 0.1% TFA in MeCN (buffer B), following this gradient program: the initial solvent condition was 45% solvent B for 5 min; the gradient was then gradually increased from 45% solvent B to 85% solvent B over 25 min. Subsequently, solvent B was increased to 100% and was kept at 100% of B for 10 min before the re-equilibration step. The semipreparative fractionation gave 14.5 mg of pure cycloprodigiosin (**2**) and 26.6 mg of pure prodigiosin (**1**). The three enriched fractions were subjected to further UPLC purification on a Phenomenex Luna 5 μm PFP column (5 μm, 4.6 mm i.d × 250 mm), with an optimized elution profile using the same solvents A and B as the mobile phases, and resulted in 2.5 mg of 5″-methyl-4″-propyl prodiginine (**3**), 0.7 mg of 11″-methyl-4″hexyl prodiginine (**4**), and 3 mg of isoheptylprodigiosin (**5**).

*Isoheptylprodigiosin (**5**):* dark pink, amorphous solid; UV (MeOH, 0.1% TFA) λ_{max} (log ε) 537 (4.3), 512 (4.0), 385 (3.1), 371 (3.1), 296 (3.3); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) Table2; HRESIMS *m/z* 352.2383 $[M + H]^+$ (calcd for $C_{22}H_{30}ON_3$, 352.2383).

Minimal Inhibitory Concentration (MIC) Assessment. The antimicrobial potential of the pure molecules was assessed by the determination of the MIC by the microdilution method and compared with appropriate antibiotics, as described by the Clinical and Laboratory Standard Institute.[46] The tests were performed in CAMHB. DMSO at an initial concentration of 2% (v/v) was used as negative control, to establish the effect on the cell growth of the solvent used to solubilize the compounds. Each compound was dissolved in DMSO and was 2-fold serially diluted from 32 to 0.015 μg/mL in a final volume of 100 μL of CAMHB, in a 96-well microtiter plate (Sarstedt). Essentially, each well contained 50 μL of test compound solution at twice the desired final concentration and was inoculated with 50 μL of bacterial seed culture grown overnight at 37 °C, yielding a final inoculum of 4×10^5 CFU/mL in a 100 μL final volume of each well. Finally, each plate was incubated for 20 h at 37 °C. The MIC_{50} has been calculated as the minimum concentration that inhibits 50% of cell population growth. The pathogens used in the screening are listed below: *S. aureus* ATCC 29213,[56] *S. aureus* ATCC 23235,[57] *S. aureus* ATCC 6538P,[49] *S. epidermidis* ATCC 35984,[58] *L. monocytogenes* MB677,[59] *S. maltophilia* ATCC 13637,[60] *S. maltophilia* ATCC 13636,[61] and *S. maltophilia* ATCC 700475.[62]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.9b01159.

Additional information (PDF)

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Notes

The authors declare no competing financial interest.

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